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Assessment of the Additional Value of Verapamil to a Moxifloxacin and Linezolid Combination Regimen in a Murine Tuberculosis Model

Elise D. Pieterman,^a Lindsey H. M. te Brake,^{b,c} Gerjo J. de Knecht,^a Aart van der Meijden,^a Jan-Willem C. Alffenaar,^d Hannelore I. Bax,^{a,e} Rob E. Aarnoutse,^b Jurriaan E. M. de Steenwinkel^a

^aDepartment of Medical Microbiology and Infectious Diseases, Erasmus Medical Centre, Rotterdam, the Netherlands

^bDepartment of Pharmacy, Radboud Institute for Health Sciences, Radboud University Medical Centre, Nijmegen, the Netherlands

^cDepartment of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, Nijmegen, the Netherlands

^dUniversity of Groningen, University Medical Centre Groningen, Department of Clinical Pharmacy and Pharmacology, Groningen, the Netherlands

^eDepartment of Internal Medicine, Section of Infectious Diseases, Erasmus University Medical Centre, Rotterdam, the Netherlands

ABSTRACT The favorable treatment outcome rate for multidrug-resistant tuberculosis (MDR-TB) is only 54%, and therefore new drug regimens are urgently needed. In this study, we evaluated the activity of the combination of moxifloxacin and linezolid as a possible new MDR-TB regimen in a murine TB model and the value of the addition of the efflux pump inhibitor verapamil to this backbone. BALB/c mice were infected with drug-sensitive *Mycobacterium tuberculosis* and were treated with human-equivalent doses of moxifloxacin (200 mg/kg of body weight) and linezolid (100 mg/kg) with or without verapamil (12.5 mg/kg) for 12 weeks. Pharmacokinetic parameters were collected during treatment at the steady state. After 12 weeks of treatment, a statistically significant decline in mycobacterial load in the lungs was observed with the moxifloxacin-linezolid regimen with and without verapamil (5.9 and 5.0 log CFU, respectively), but sterilization was not achieved yet. The spleens of all mice were culture negative after 12 weeks of treatment with both treatment modalities, and the addition of verapamil caused a significant reduction in relapse (14/14 positive spleens without versus 9/15 with verapamil, $P = 0.017$). In conclusion, treatment with a combination regimen of moxifloxacin and linezolid showed a strong decline in mycobacterial load in the mice. The addition of verapamil to this backbone had a modest additional effect in terms of reducing mycobacterial load in the lung as well as reducing the spleen relapse rate. These results warrant further studies on the role of efflux pump inhibition in improving the efficacy of MDR-TB backbone regimens.

KEYWORDS *Mycobacterium tuberculosis*, efflux pump inhibitor, linezolid, moxifloxacin, verapamil

Tuberculosis (TB) is the leading cause of death from infectious disease worldwide. An estimated 1.7 million people died due to this disease in 2016, and an estimated 10.4 million people fell ill in the same year (1). Drug resistance remains a problem for the treatment of TB; an estimated 4% of the new TB cases and 19% of previously treated TB cases had rifampin-resistant or multidrug-resistant TB (MDR-TB) (1). Currently, patients with MDR-TB need to undergo up to 2 years of treatment, which is often associated with toxic side effects, while only 54% of patients are being cured (1). Novel

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Address correspondence to Elise D. Pieterman, e.pieterman@erasmusmc.nl.

treatment regimens that are more effective, short, and safe need to be developed to treat this devastating disease.

In order to increase the treatment efficacy in the short term, we focused in this study on drugs which are already registered for use. Fluoroquinolones, in particular, moxifloxacin (MXF) and levofloxacin, are considered to be among the most important groups of drugs in the treatment of MDR-TB (2, 3). In murine TB models moxifloxacin was found to be the most bactericidal of all quinolones. It also showed good activity against rifampin-tolerant TB and MDR-TB in different mouse TB models (4, 5). Furthermore, a randomized controlled trial showed promising success rates in patients with MDR-TB treated with a regimen that included moxifloxacin (6). Therefore, moxifloxacin is now recommended by the World Health Organization (WHO) in their most recent guideline outlining a shorter MDR-TB regimen (8 to 12 months instead of at least 18 months of treatment) for patients who were not previously treated with second-line drugs (7).

In the current WHO treatment guideline, linezolid (LZD) (an oxazolidinone) is classified as a core second-line agent (group C) (7). *In vitro* and *in vivo* studies showed good activity of linezolid against multiple MDR-TB strains (4, 8), and a systematic review and meta-analysis of patients treated with linezolid-containing MDR-TB and extensively drug-resistant TB (XDR-TB) regimens showed promising results (9). Furthermore, a meta-analysis of MDR-TB patients treated with (at that time) group 5 drugs showed that the use of linezolid increased the probability of a successful treatment outcome in this group by 57% (10). Ongoing studies include an evaluation of linezolid combined with bedaquiline and pretomanid in a short (6-month) trial of treatment for XDR-TB (Nix-TB) (11) and a phase 3 study evaluating various doses and treatment durations of linezolid, bedaquiline, and pretomanid in MDR-TB and XDR-TB patients (ZeNix trial; ClinicalTrials registration no. NCT03086486).

Although the effectiveness of moxifloxacin and linezolid appears to be promising, the emergence of resistance to both drugs is a problem. There is accumulating evidence supporting a role for mycobacterial efflux pumps in the extrusion of TB drugs and emergence of drug resistance to both fluoroquinolones and oxazolidinones (12, 13). Thus, inactivation of these mycobacterial efflux pumps by efflux pump inhibitors (EPIs) could be a valuable strategy to increase intrabacterial drug concentrations and reduce the emergence of drug resistance (14).

Verapamil was the first discovered inhibitor of P-glycoprotein-mediated drug efflux (15) and was shown to be able to increase the accumulation of P-glycoprotein substrate drugs in macrophages (16). Another study showed that macrophage-induced tolerance of moxifloxacin could be reversed upon exposure to verapamil (12). Furthermore, verapamil not only affects macrophage efflux pumps but also has been identified as an inhibitor of *Mycobacterium tuberculosis* efflux systems (17–20). Besides, verapamil is widely used for the treatment of cardiovascular diseases, in contrast to several other EPIs which are not registered yet or have been withdrawn from the market due to severe side effects (21–23).

In the present study, we evaluated the value of the addition of verapamil to a backbone of moxifloxacin and linezolid. We assessed the efficacy of this regimen in BALB/c mice infected with a *M. tuberculosis* strain of the Beijing genotype and evaluated the pharmacokinetic profiles of drugs in this regimen.

RESULTS

Mycobacterial load (efficacy) assessment. Both moxifloxacin plus linezolid (group 1) and moxifloxacin plus linezolid plus verapamil (group 2) combination treatments were well tolerated, although mice showed mild distress during the first 3 weeks of treatment in both groups.

The mycobacterial load in lungs and spleen during 12 weeks of treatment and after a 12-week posttreatment period is presented in Fig. 1. The median total amount of mycobacteria in the lungs at start of treatment was 8.18 log CFU (interquartile range [IQR], 7.96 to 8.19). During the 12 weeks of treatment, the mycobacterial load in the

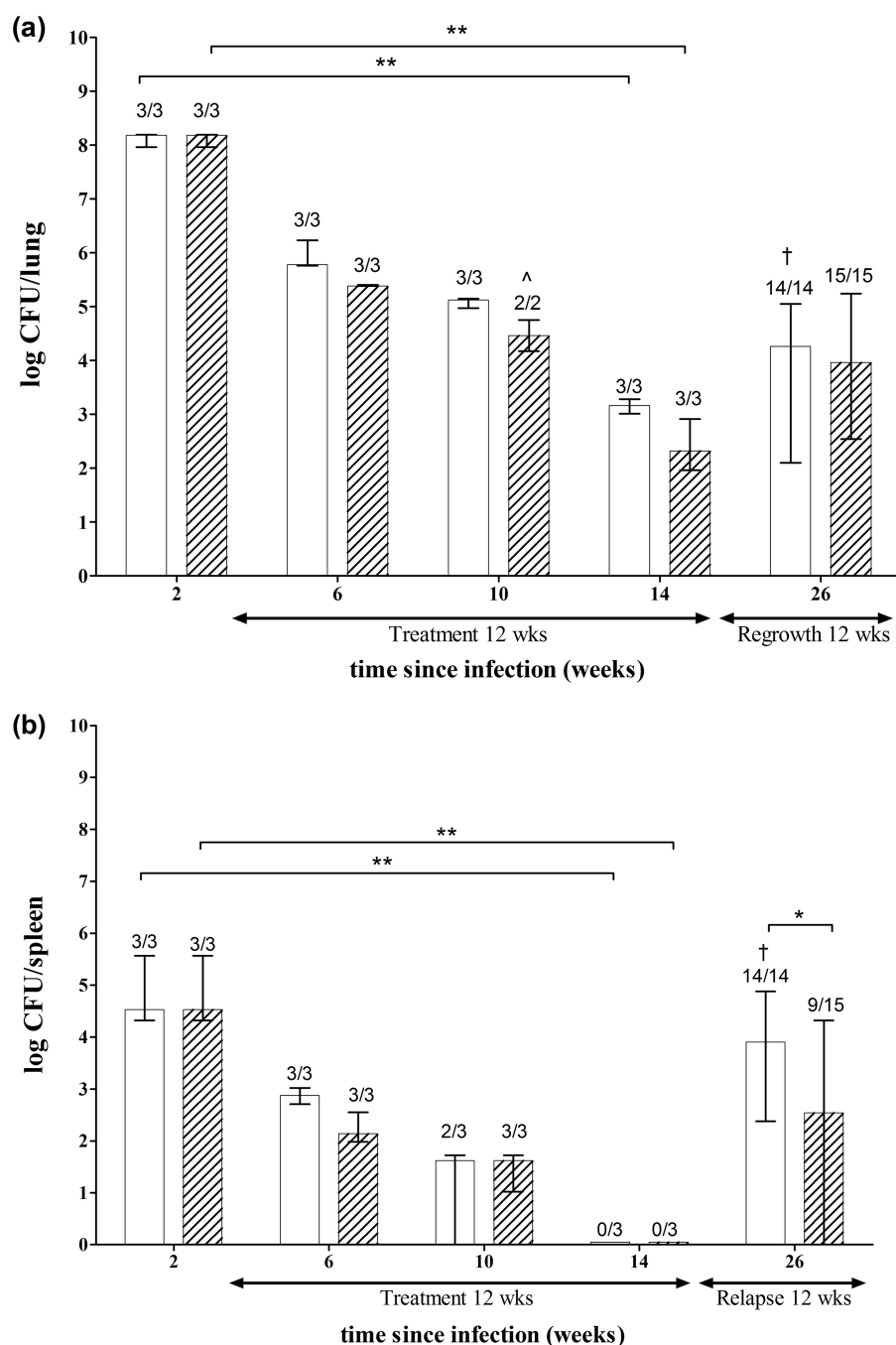


FIG 1 Mycobacterial load in lung (a) and spleen (b) expressed as median \pm range (error bars) of the CFU per organ, at weeks 2, 6, 10, and 14 and at 12 weeks posttreatment (week 26). Gray bars represent mice treated with moxifloxacin and linezolid; striped bars represent mice treated with moxifloxacin, linezolid, and verapamil. Numbers above bars represent the numbers of culture-positive mice among the total numbers of mice at that time point. Significance is noted as follows: **, $P < 0.0001$; *, $P < 0.05$. †, 1 mouse became moribund before the planned dissection; ^, 1 measurement is missing due to a contaminated plate. LZD, linezolid; MXF, moxifloxacin; VPM, verapamil.

lungs declined, with 5.0 log CFU in group 1 ($P < 0.0001$) and with 5.9 log CFU in group 2 ($P < 0.0001$) compared to the start of treatment. No significant difference between group 1 and group 2 was observed at this time point. No sterilization of the lungs was achieved with either of the treatment modalities. As a result, no assessment of relapse in lung tissue was possible. In contrast, the spleens of all mice were culture negative after 12 weeks of treatment. The addition of verapamil was associated with a significant

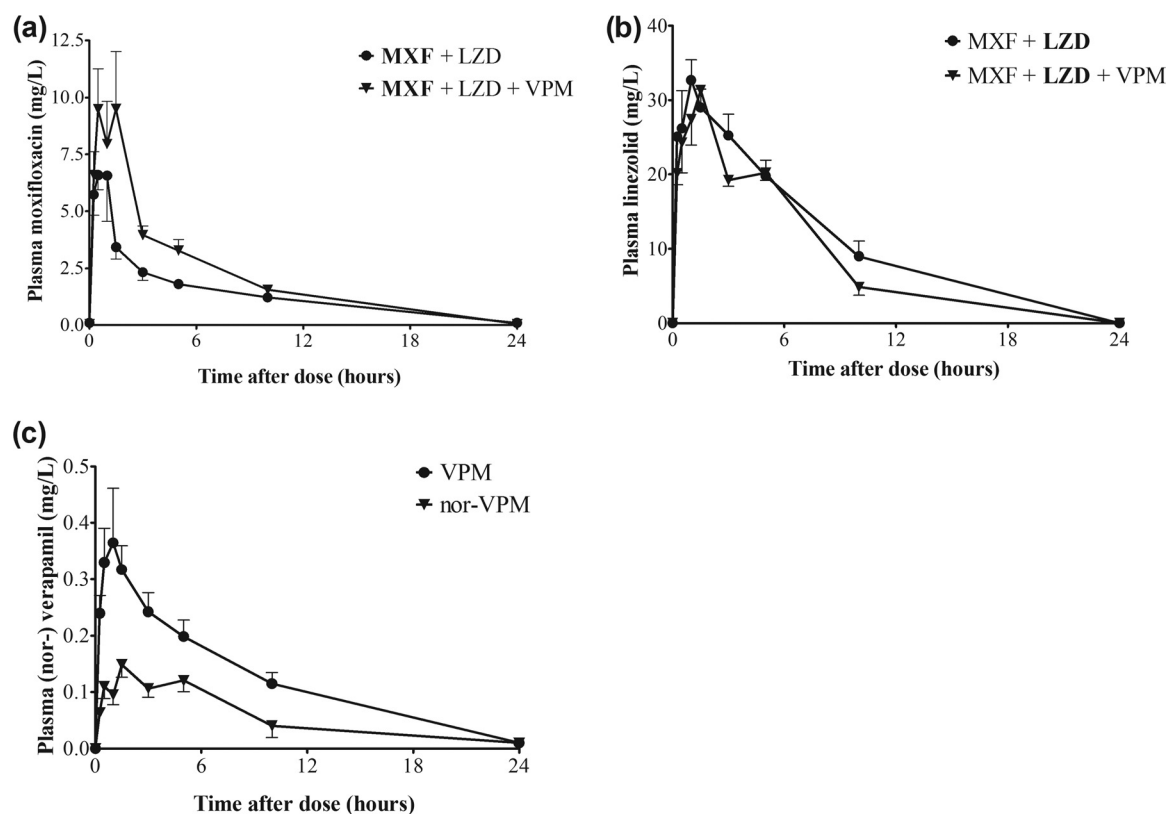


FIG 2 Moxifloxacin (a), linezolid (b), and (nor)verapamil (c) plasma concentration-time profiles at steady state following an oral dose of 200 mg/kg moxifloxacin, 100 mg/kg linezolid, and/or 12.5 mg/kg verapamil in *M. tuberculosis*-infected BALB/c mice. Plasma concentrations are plotted as means \pm standard errors of the means (SEM) of results from three mice per study drug per time point. LZD, linezolid; MXF, moxifloxacin; VPM, verapamil; nor-VPM, norverapamil.

reduction in relapse after 3 months of treatment, as measured in the spleen (9/15 culture-positive samples in group 2 versus 14/14 in group 1, $P = 0.017$).

Pharmacokinetic evaluation. Plasma concentration-time profiles and pharmacokinetic parameters of the study drugs are shown in Fig. 2 and Table 1.

The total exposure to moxifloxacin was 53% higher in the mice that received verapamil; also, the peak concentration (C_{\max}) was 44% higher with addition of verapamil (9.5 versus 6.6 mg/liter). In contrast, the area under the plasma concentration-time curve from 0 to 24 h (AUC_{0-24}) of linezolid was slightly (17%) lower

TABLE 1 Pharmacokinetic parameters at steady state of moxifloxacin and linezolid and (nor)verapamil in plasma following oral administration in *M. tuberculosis*-infected BALB/c mice^a

Drugs	Dose (mg/kg)	$AUC_{0-\tau}$ (mg/liter \cdot h)	C_{\max} (mg/liter)	Cl/F (liters/h)	V/F (liters)	$t_{1/2}$ (h)
MXF (+ LZD)	200	30.0	6.6	0.15	0.92	4.3
MXF (+ LZD + VPM)	200	46.0	9.5	0.10	0.43	3.1
LZD (+ MXF)	100	215	32.7	0.010	0.028	1.9
LZD (+ MXF + VPM)	100	179	31.3	0.012	0.035	1.9
VPM (+ MXF + LZD)	12.5	2,670 ^b	365 ^b	0.00010	0.00067	4.5
nor-VPM (+ MXF + LZD)	NA	1,387 ^b	149 ^b	NA	NA	NA

^aPharmacokinetic parameters are based on 8 time points; concentrations at each time point are based on plasma samples of 3 mice. $AUC_{0-\tau}$, area under the plasma concentration-time curve within the dosing interval; Cl, clearance; C_{\max} , maximum plasma concentration; F, bioavailability; LZD, linezolid; MXF, moxifloxacin; NA, not applicable; nor-VPM, norverapamil; $t_{1/2}$, half-life; V, volume of distribution; VPM, verapamil.

^bFor verapamil and norverapamil, $AUC_{0-\tau}$ and C_{\max} are expressed in micrograms per liter per hour and micrograms per liter, respectively.

in mice that received verapamil, whereas the linezolid peak concentrations in the two groups were nearly identical (31.3 and 32.7 mg/liter). The total exposures of verapamil and its active metabolite norverapamil were 2,670 $\mu\text{g/liter} \cdot \text{h}$ and 1,387 $\mu\text{g/liter} \cdot \text{h}$, respectively, with C_{max} levels of 365 $\mu\text{g/liter}$ and 149 $\mu\text{g/liter}$, respectively.

DISCUSSION

To our knowledge, this was the first study evaluating whether verapamil could increase the treatment efficacy of a combination regimen consisting of moxifloxacin and linezolid in a murine TB model. Previous studies examining the additional value of verapamil in the treatment of TB focused on combining verapamil with only one drug or with the standard drug regimen against drug-sensitive *M. tuberculosis* (24–26), whereas our study focused on improving a new two-drug MDR-TB backbone.

In our study, the combination of moxifloxacin and linezolid achieved a strong reduction in mycobacterial load in the lungs after 3 months of treatment and the mycobacterial load in the spleen was undetectable at that time point. Although multiple randomized controlled trials showed promising treatment outcomes of MDR-TB regimens containing linezolid or moxifloxacin, with success rates of approximately 80%, limited data are available on the combination of the two drugs in humans (6, 27). Most studies on MDR-TB regimens containing linezolid in humans were performed either in the presence of moxifloxacin resistance or with linezolid added to a wide variety of different TB drugs (28, 29). However, previous *in vitro* studies showed that combining the two drugs resulted in synergistic as well as indifferent or antagonistic effects, which has been postulated to be strain dependent (30, 31).

In the present study, the addition of verapamil did not significantly increase the treatment efficacy of the moxifloxacin-linezolid backbone in terms of treatment outcome at 3 months of treatment. However, we did observe a modest additional decline in mycobacterial load in the lungs at the end of treatment as well as a significantly reduced relapse rate in the spleen with the addition of verapamil. Several other studies showed promising effects of the use of verapamil in combination with different anti-TB drugs. For example, Gupta et al. showed that verapamil could increase the treatment efficacy of a standard TB drug regimen consisting of rifampin, isoniazid, and pyrazinamide in mice infected with drug-sensitive *M. tuberculosis* (24). Similarly, it was shown for the new TB drug bedaquiline that subinhibitory dosing of bedaquiline in a murine drug-sensitive TB model could become as effective as regular dosing after the addition of verapamil (25). Both murine TB studies showed a significant difference of ± 1 log CFU between regimens with and without verapamil at different time points (24, 25). This observation was comparable to our findings in the lung tissue. Also in line with the results of our study showing a reduced relapse rate in the spleen, Gupta et al. showed reduced relapse rates with the addition of verapamil to the standard TB drug regimen (24). However, since all mice in our study were still culture positive in the lungs, it is difficult to assess the clinical value of this observation. Therefore, it would be worthwhile to evaluate the efficacy of this regimen in a future study for an extended period of time, in order to assess whether sterilization could be achieved in all tissues. In that case, relapse reduction mediated by verapamil, as observed by Gupta et al. (24), might possibly be replicated and might support the idea of the value of the addition of verapamil to this backbone. Interestingly, our previous *in vitro* study did not show any additional effect of verapamil on the activity of the moxifloxacin-linezolid combination (32). It could be hypothesized that the effect observed in the present study was mediated through the effects of verapamil on the efflux pumps present in macrophages. Such an effect was shown by Adams et al. in a TB macrophage model where the addition of verapamil reduced tolerance to multiple TB drugs (12).

In order to evaluate the influence of verapamil on the pharmacokinetics of our backbone, we compared the pharmacokinetic parameters of moxifloxacin and linezolid with and without verapamil. We observed that the total exposure to moxifloxacin was approximately 53% higher upon the addition of verapamil. These results were comparable to the observations of Xu et al., who found a 46% increased AUC of bedaquiline

after addition of verapamil (26). The authors postulated that the increased effectivity of bedaquiline was likely due to the increased bioavailability mediated through an effect of verapamil on mammalian drug transporters rather than to inhibiting efflux pumps present in mycobacteria and/or macrophages (26). Since moxifloxacin is subject to P-glycoprotein-mediated efflux (33), the increased AUC could perhaps be ascribed to the inhibition of P-glycoprotein-mediated drug efflux at the site of the intestines of the mice (34) and might have been the cause of the modest decline in CFU in the lungs. Besides, it is unlikely that the increased effectivity of the backbone after verapamil addition was due to the antimycobacterial effect of verapamil only, since in our previous *in vitro* study verapamil showed (modest) activity only at concentrations of ≥ 64 mg/liter (32). Moreover, other *in vivo* studies also showed no effect of the verapamil for single drug exposures (20, 26).

The results of our pharmacokinetic analyses confirm that drug exposures in this murine study resemble those achieved in humans. Total exposures to moxifloxacin (AUC within the dosing interval $[AUC_{0-t}]$, 30 mg/liter \cdot h) were comparable with the range of exposures found in humans (AUC_{0-24} , 25 to 29 mg/liter \cdot h), which were obtained with the recommended 400 mg of moxifloxacin orally (35, 36). Similarly, total exposures to linezolid in our study were comparable to AUC_{0-12} values found in pharmacokinetic studies in humans after an oral intake of 600 mg linezolid twice daily (AUC_{0-12} , 108 to 146 mg/liter \cdot h) (37, 38). Verapamil exposure in our study mimicked the range observed in humans, namely, an AUC_{0-24} of 3,253 μ g/liter \cdot h after daily oral intake of 240 mg of slow-release verapamil (39) and an AUC_{0-8} of 1,999 μ g/liter \cdot h after thrice-daily oral intake of 120 mg immediate-release verapamil with a norverapamil total exposure of 2,312 μ g/liter \cdot h (40). Although total exposures were comparable to human exposures, the C_{max} levels of moxifloxacin and linezolid in this study were higher than those seen in humans (6.6 mg/liter versus 3.9 mg/liter and 32.7 mg/liter versus 20.4 mg/liter, respectively) (36, 37). Since the AUC/MIC ratio is considered to be the driver of efficacy for moxifloxacin and linezolid (41, 42) and the moxifloxacin C_{max} /MIC ratio showed a poor correlation in efficacy in BALB/c mice (42), we assumed that this has a limited effect on the translational value.

In conclusion, the present study in mice showed a strong and steady CFU decline with the combination of moxifloxacin and linezolid. The addition of verapamil had a modest additional effect in terms of reducing mycobacterial load in the lung after 3 months of treatment as well as in terms of reducing spleen relapse rates. These results warrant further studies on the role of efflux pump inhibition in improving the efficacy of MDR-TB backbone regimens.

MATERIALS AND METHODS

Animals. Female BALB/c mice that were specified to be free of pathogens were obtained from Charles River (Les Oncins, France). At the start of the experiments, animals were 13 to 15 weeks old and weighed 20 to 25 g. Experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act and were in concordance with EU animal directive 2010/63/EU. The Institutional Animal Care and Use Committee of the Erasmus Medical Centre (MC) approved the present protocols (117-12-14).

Bacterial strain. The *M. tuberculosis* Beijing VN 2002-1585 genotype strain (43), with a moxifloxacin MIC of 0.25 mg/liter and a linezolid MIC of 1 mg/liter, was used (43, 44). MICs were determined according to Clinical and Laboratory Standards Institute (CLSI) standards (45).

Experimental setup. For treatment efficacy and pharmacokinetic analyses, a total of 99 mice (51 mice for efficacy assessment and 48 mice for pharmacokinetic analysis) were infected with *M. tuberculosis* as described previously (46). In short, mice under anesthesia were infected by intratracheal instillation with a suspension (40 μ l) containing 2.9×10^5 CFU (2.5×10^5 to 3.2×10^5) of *M. tuberculosis*, followed by inhalation to ensure the formation of a bilateral TB infection (46). Two weeks after infection, three mice were used as controls to determine infection efficacy and reproducibility over time. A total population of 48 mice was divided in two combination therapy groups; the first group received 200 mg moxifloxacin/kg of body weight (Bayer, Leverkusen, Germany) plus 100 mg/kg linezolid (Sigma-Aldrich, Zwijndrecht, the Netherlands), and the second group received the same backbone in combination with 12.5 mg/kg verapamil (Sigma-Aldrich, Zwijndrecht, the Netherlands). Mice were treated for 12 weeks. After 12 weeks of treatment, 30 mice ($n = 15$ per group) were kept for another 12 weeks without treatment to assess relapse in lung and spleen. Selected doses were chosen based on previous *in vivo* mouse studies (25, 47, 48). Dry powder moxifloxacin was dissolved in distilled water plus 0.05% agarose using a mortar and pestle. Dry powder linezolid was dissolved in distilled water and added to the moxifloxacin suspension. Verapamil was dissolved in distilled water and added to the moxifloxacin-plus-

linezolid suspension. Drugs were administered orally 5 times per week, using a feeding cannula, in a total volume of 0.2 ml per day.

Mycobacterial load (efficacy) assessment. A total of 51 of the infected mice receiving treatment were sacrificed at the start of therapy or after 4, 8, and 12 weeks of therapy ($n = 3$ per time point per regimen) and at 12 weeks posttreatment to assess relapse ($n = 15$ per regimen). Relapse was defined as the presence of ≥ 1 CFU on a culture plate, observed after complete sterilization at 12 weeks of treatment. To prevent carryover of TB drugs, therapy was stopped 72 h before the mice sacrificed and activated charcoal (0.4%) was added to the culture media. The lungs and spleen were removed aseptically and homogenized separately in M-tubes using a gentleMACS Octo Dissociator and the RNA program (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 ml phosphate-buffered saline. Serial 10-fold dilutions were performed with each tissue homogenate, and samples of 200 μ l were cultured on drug-free 7H10 Middlebrook agar containing activated charcoal and incubated for 28 days at 37°C with 5% CO₂ to perform colony counting.

Pharmacokinetic analyses. Pharmacokinetic analyses were performed at the steady state after 4 weeks of treatment in the other population of the infected mice ($n = 48$). These mice were sacrificed by CO₂ exposure, and blood samples were taken via cardiac puncture at 0.25, 0.5, 1, 1.5, 3, 5, 10, and 24 h after the dose. Three animals were euthanized for each of the eight sampling time points in each group ($n = 48$ total). Blood was collected in microcentrifuge tubes containing EDTA. Subsequently, blood was centrifuged at 10,000 $\times g$ for 5 min to obtain plasma, which was stored at -80°C upon analysis.

Moxifloxacin concentrations in plasma were analyzed with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay, validated for human plasma, at the University Medical Center Groningen, the Netherlands (49). Cross-validation of the assay for human plasma to murine plasma was performed by comparing responses of 5-fold measurements of quality control (QC) moxifloxacin samples (at concentrations of 0.5, 2.5, and 5.0 mg/liter) in human plasma with those determined in murine plasma after analysis of all samples with the LC-MS/MS assay for human plasma. The accuracy of measurement of murine samples with the assay for human samples was between 88% and 97%, and the within-run coefficient of variation (CV) amounted to 4.0% to 6.2%.

Linezolid concentrations in plasma were analyzed by a validated high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay at the University Medical Center Groningen, Groningen, the Netherlands (50). The assay accuracy was 96.3% to 108.5%, the within-run CV ranged from 2.5% to 7.1%, and the lower limit of quantitation (LLOQ) was 0.05 mg/liter. Cross-validation between human and murine plasma matrices was performed by comparing the responses seen with three QC linezolid concentrations (0.5, 15, and 30 mg/liter in 5-fold) in human plasma with those in murine plasma. The accuracy of murine plasma measurements relative to human plasma measurements was between 101% and 114%, with a within-run CV of 1.7% to 2.6%.

Verapamil and norverapamil concentrations were analyzed on a Thermo Fisher (San Jose, CA, USA) triple-quadrupole LC-MS/MS system with a Finnigan Surveyor LC pump and a Finnigan Surveyor autosampler. The mobile phase consisted of an aqueous buffer (containing ammonium acetate at 5 g/liter, acetic acid at 35 ml/liter, and trifluoroacetic anhydride at 2 ml/liter water), water, and acetonitrile and had a flow rate of 0.3 ml/min. Samples were prepared by the use of 100 μ l serum or plasma and 750 μ l precipitation reagent (mixture of methanol and acetonitrile [4:21 {vol/vol}]) containing cyanomipramin as an internal standard, subjected to vortex mixing for 1 min, and subsequently centrifuged at 11,000 $\times g$ for 5 min. From the clear upper layer, 5 μ l was injected onto the LC-MS/MS system. A Finnigan TSQ Quantum Discovery mass selective detector was used in electrospray positive ionization mode and performed selected reaction monitoring. The mass transitions for verapamil were 455.3 m/z to 165.1 m/z and for norverapamil were 441.2 m/z to 165.0 m/z ; a scan width of 0.5 m/z was used. The calibration curves were linear within the concentration range of 18 to 1,800 μ g/liter for verapamil and 19 to 1,900 μ g/liter for norverapamil, with correlation coefficients (R^2) of 0.997 and 0.999, respectively. This method was precise and accurate; within-day precision ranged between 1.0% and 5.5% for verapamil and between 1.6% and 5.9% for norverapamil, and between-day precision ranged from 1.5% to 3.1% for verapamil and 0.0% to 1.2% for norverapamil. The calculated accuracy ranged from 1.8% to 4.5% for verapamil and 0.2% to 2.0% for norverapamil.

All measured concentrations represent total (i.e., protein-bound plus unbound) drug concentrations. Pharmacokinetic parameters were assessed using standard noncompartmental methods in Phoenix WinNonlin version 6.4 (Pharsight Corporation), as described previously (51).

Statistical analysis. CFU counts were log10 transformed before analysis. Group mean CFU counts at various time points after start of treatment were compared using one-way analysis of variance with a Bonferroni multiple-comparison test. Proportions of mice relapsing were compared using Fisher's exact test. In a previous study, we observed that a sample size of 3 or more mice is sufficient to achieve 100% power to detect a statistically significant difference in potency of 50% between different treatments (52). The statistical significance level adopted was a P value of <0.05 . Analyses were performed using Prism 5 (GraphPad Software, San Diego, CA, USA).

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We have no conflicts of interest to declare.

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